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Regulating the regulator: nitric oxide control of post-translational modifications

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SUMMARY

Nitric oxide (NO) is perfectly suited for duties as a redox signalling molecule. A key route for NO bioactivity occurs via protein *S*-nitrosation, the addition of a NO moiety to a protein cysteine (Cys) thiol (-SH) to form a *S*-nitrosothiol (SNO). This process is thought to underpin a myriad of cellular processes in plants linked to development, environmental responses and immune function. Here we collate emerging evidence showing that NO bioactivity regulates a growing number of diverse post-translational modifications (PTMs) including SUMOylation, phosphorylation, persulfidation and acetylation. We provide examples of how NO orchestrates these processes to mediate plant adaptation to a variety of cellular cues.

Introduction

More than two hundred reversible protein post-translational modifications (PTMs) have been identified to date, massively expanding the proteome and by extension enabling a plethora of protein functions (Minguez *et al.*, 2012), providing an escape from genetic incarceration. Typically, PTMs target amino acid residues embedded within conserved motifs (Tompa *et al.*, 2014). In this context, redox signalling is rapidly emerging as a key regulator of plant protein function associated with a myriad of plant processes. The small gaseous molecule, nitric oxide (NO), is a central player in redox signal transmission, mediating its redox functions predominantly through *S*-nitrosation / *S*-nitrosylation: the addition of a NO moiety to a cysteine (Cys) sulfhydryl/thiol to form an *S*-nitrosothiol (SNO) (Lindermayr *et al.*, 2005; Besson-Bard *et al.*, 2008b; Leterrier *et al.*, 2011; Yun *et al.*, 2016). This redox-based modification has been shown to regulate development, environmental responses and plant immunity. The emerging evidence suggests that NO orchestrates some of these processes through regulating the deployment of diverse PTMs. Here, we highlight some of these recent developments.

SUMOylation

SUMOylation, the covalent attachment of the small ubiquitin-like modifier (SUMO) to target proteins is emerging as a key modulator of eukaryotic immune function. In plants, a SUMO1/2-dependent process has been proposed to control the deployment of host immunity (Lee *et al.*, 2008a; van den Burg *et al.*, 2010; Saleh *et al.*, 2015). Recently, a key role for *S*-nitrosation in the control of SUMOylation has emerged (Skelly *et al.*, 2019). Following the pathogen triggered nitrosative burst, increasing NO levels were shown to drive *S*-nitrosation of the *Arabidopsis* SUMO E2 enzyme, SCE1, at Cys139. The SUMO-conjugating activities of both SCE1 and its human homologue, UBC9, were both blunted by this PTM (Fig. 1a). Accordingly, mutation of Cys139 resulted in accumulation of SUMO1/2 conjugates (Fig. 1b), disabled immune responses and increased pathogen susceptibility (Skelly *et al.*, 2019). Collectively, these findings establish that *S*-nitrosation of SCE1 at Cys139 enables NO bioactivity to promote immune activation by relieving SUMO1/2-mediated suppression. This discovery is important because it suggests a new paradigm for the regulation of SUMOylation. The global control of this PTM is predominantly thought to occur at the level of each substrate via complex local machineries (Bossis & Melchior, 2006). In contrast, these new findings uncover a novel, parallel and complementary mechanism by establishing that total SUMO conjugation is additionally regulated directly by SNO formation at SCE1 Cys139. Significantly, this Cys

residue is evolutionary conserved and specifically *S*-nitrosated in human UBC9, implying this immune-related regulatory process might be conserved across phylogenetic kingdoms (Skelly *et al.*, 2019). Thus, NO bioactivity conveyed through *S*-nitrosation is a key regulator of SUMOylation, a ubiquitous eukaryotic PTM.

Phosphorylation

The emerging data suggests that NO is also a major regulator of phosphorylation-dependent signalling cascades. NO accumulation can trigger the activation of protein kinases (PKs) as well as the phosphorylation of numerous proteins related to diverse cellular processes (Besson-Bard *et al.*, 2008a; Frederickson Matika & Loake, 2014; Del Castello *et al.*, 2019). NO-dependent PKs include Ca^{2+} -dependent PKs (CDPKs), sucrose non-fermenting 1-related PKs (SnRKs), mitogen-activated PKs (MAPKs) and phosphoinositide-dependent PKs (PDKs). However, the mechanism(s) by which NO modulates the activity of these target PKs remains unclear.

NO is thought to mediate the activation of MAPKs and CDPKs indirectly through the mobilization of cytosolic free Ca^{2+} (Besson-Bard *et al.*, 2008b). Yet, the subtle mechanisms underlying this process also remain to be determined. Direct *S*-nitrosation has not been confirmed for SnRKs (Wawer *et al.*, 2010), nor reported for MAPKs or CDPKs. However, the activity of the tomato cell-death regulator PDK1 was found to be inhibited by *S*-nitrosation of a critical catalytic Cys residue. Additionally, the activity of MAPKs may be modulated by tyrosine nitration as suggested by preliminary experiments (Ling *et al.*, 2012). Indeed, MAPKs become activated by MAPK kinases (MAPKKs) through the dual phosphorylation of a Thr-X-Tyr motif in the activation loop. It is therefore tempting to speculate that nitration of the Tyr residue within the activation loop could interfere with its phosphorylation by MAPKKs and, consequently, negatively modulate MAPK activity.

Finally, NO might modulate phosphorylated PK and, more generally, phosphorylated proteins through the redox regulation of protein phosphatases (PPs). This process is well established in animals and affects major phosphatases, including tyrosine phosphatases (Nakamura & Lipton, 2019). In this context, either activation, inhibition or a protective effect of the PP against oxidation-induced inactivation have been observed, depending on the specific PP. However, to

date, no NO-dependent PP have been characterized in plants. So, this would be an interesting area for future exploration.

More generally, it is tempting to speculate that the post-translational modification of residues by NO or NO-derived compounds could trigger steric hindrance altering the interaction with and phosphorylation by upstream kinases. For instance, S-nitrosation of the phosphotransfer protein AHP1, involved in cytokinin signalling, suppresses its phosphorylation, repressing cytokinin signalling (Feng *et al.*, 2013). The reciprocity of this mechanism could be also possible: phosphorylation of a given protein could also impact its subsequent S-nitrosation.

Histone acetylation and methylation

Chromatin structure in eukaryotic organisms is very dynamic and is altered during growth and development and in response to environmentally stimuli. Modification of histone proteins induces chromatin remodelling to control transcription, replication, recombination and repair (Bannister & Kouzarides, 2011). Adjustment of histone acetylation or methylation, catalyzed by histone acetyltransferases / histone deacetylases (HDAs) and methyltransferases / demethylases, respectively, are integral to these processes (Servet *et al.*, 2010; Shen *et al.*, 2015). Recently, it has been demonstrated that NO affects histone acetylation by targeting and inhibiting histone deacetylase (HDA)-complexes (Mengel *et al.*, 2017). Genome-wide NO-dependent H3K9/14ac profiling in *Arabidopsis* seedlings identified NO-regulated histone acetylation of genes integral to immunity, abiotic stress and chloroplast function, suggesting that NO bioactivity might regulate gene expression by the modulation of chromatin structure (Mengel *et al.*, 2017). A direct effect of NO on enzymes catalyzing DNA or histone methylation/de-methylation in plants has not been reported. However, genes encoding these enzymes are induced by NO or differentially expressed in plants with impaired NO homeostasis (Shi *et al.*, 2014; Hussain *et al.*, 2016; Kovacs *et al.*, 2016). Moreover, NO accumulation has been shown to induce global DNA hypomethylation, resulting in altered expression of chromatin remodeling enzymes (Ou *et al.*, 2015). This implies an indirect effect of NO on chromatin methylation mechanisms in plants. In aggregate, the emerging data suggests NO bioactivity might play important roles in the nucleus, however, the molecular details still require further investigation.

Crosstalk between NO, ROS and H₂S

Nitro-fatty acids are reactive signaling mediators that are formed when unsaturated fatty acids, typically oleic or oleic acid, react with NO or reactive nitrogen species (RNS) (Kelley *et al.*, 2008; Corpas *et al.*, 2013). Recently, nitro-oleic acid has been found to activate NADPH oxidase (RBOH) altering reactive oxygen species (ROS) production (Arruebarrena *et al.*, 2020) which implies a novel signal link between NO- and ROS-based signalling. It is already well established that the isoenzyme RBOH is S-nitrosated at Cys890 inhibiting the activity of this enzyme and thus curbing the pathogen-triggered oxidative burst to limit the extent of HR associated cell death (Yun *et al.*, 2011). Additionally, the main enzymatic source of peroxisomal hydrogen peroxide (H₂O₂), glycolate oxidase, is also inactivated by S-nitrosation (Ortega-Galisteo *et al.*, 2012) and possibly also nitration (Lozano-Juste *et al.*, 2011), suggesting dual NO-dependent regulation. NO-based PTMs may also affect several ROS scavenging enzymes and some of these, for example, ascorbate peroxidase (APX) and superoxide dismutase (SOD), were found to be inversely regulated by S-nitrosation and nitration (Yang *et al.*, 2015; Kolbert & Feigl, 2017). Thus, NO-related PTMs may act as an on-off switch for antioxidant enzyme activities.

In addition to NO, hydrogen sulphide (H₂S) and H₂O₂ are also recognized as redox signal molecules in both animal and plant cells. They can also affect protein function through their redox interactions with critical thiols (-SH) on side groups of Cys residues, leading to PTMs. H₂O₂ causes oxidation of cysteinyl thiols to sulphenic acid, also identified as S-sulfenylation (Huang *et al.*, 2019), whilst H₂S results in persulfidation (Hancock, 2019; Corpas *et al.*, 2019b). Surprisingly, many of the targets for these molecules are key enzymes involved in ROS metabolism (Table 1).

In aggregate, the emerging evidence suggests that NO-related PTMs modulate enzymes involved in both ROS production and scavenging, suggesting that NO tightly regulates ROS homeostasis. Beyond direct protein modifications, NO may also compete for direct targets of both ROS and H₂S-based PTMs, indicating the possibility of multi-level regulation.

NO regulation of the N-end rule protein degradation pathway

Transcriptional responses to reduced oxygen (hypoxia) are achieved by oxygen-dependent degradation by the ubiquitin proteasome system (UPS) of transcription factors mediated through the N-end rule (Gibbs *et al.*, 2016; Dissmeyer *et al.*, 2018). This pathway of targeted proteolysis relates the stability of a protein to the nature of its N-terminus. The Arginine (Arg) branch of the N-end rule results in the exposure of Cys at the N-terminus which can undergo *S*-nitrosation or oxidation to sulphenic or sulphonic acid, triggering arginylation of the target protein by arginyl-tRNA transferases (ATEs). These enzymes transfer Arg from Arg-tRNA to the N α -amino group of the Nt residue leading to N-recognin-mediated ubiquitination and subsequent degradation (Varshavsky, 2011).

Group VII ethylene response factors (ERFs) are important regulators of oxygen sensing as they become substrates of the N-end rule pathway. Significantly, group VII ERFs are also degraded in the presence of NO and oxygen and may thus serve as NO and oxygen sensors regulating NO function in a number of developmental processes (Gibbs *et al.*, 2014). Thus, oxygen sensing during hypoxia (reduced oxygen levels), occurring, for example, in flooded roots, also requires low levels of NO in order to stabilise group VII ERFs, which orchestrate cellular responses ameliorating the impact of hypoxia. Under hypoxia, as the oxygen level decreases typically NO levels increase (Gupta *et al.*, 2005), presenting a problem. It has recently been shown that ethylene can enhance ERFVII stability prior to hypoxia by increasing the NO-scavenger Phytooglobin1 (Hartman *et al.*, 2019). This ethylene-mediated NO depletion and consequent ERFVII accumulation might enable pre-adaptation of plants prior to hypoxia. In aggregate, the emerging findings suggest that NO-dependent modification of sentinel proteins embedded within the N-end rule protein degradation pathway may under some circumstances enable NO perception, while depletion of this molecule by Phytooglobin1 supports pre-adaptation to hypoxia.

NO regulation of methylation linked to pre-mRNA splicing

Recently, a novel mechanism of NO crosstalk with protein arginine methylation, a common post-translational modification that regulates multiple biological processes (Fuhrmann *et al.*, 2015) has been identified in plant stress responses (Hu *et al.*, 2017). Arginine methyltransferases (PRMTs), utilize S-adenosyl-L-methionine as a donor of the methyl group

transferred to target arginine residues. PRMTs play wide roles in the biology of the cell including pre-mRNA splicing and mRNA translation (Blanc & Richard, 2017). Plant PRMTs are known to control key developmental processes including growth, flowering, the circadian cycle and also response to salinity (Ahmad & Cao, 2012). Among nine PRMT families, PRMT5 is localized to both the nucleus and cytoplasm and is one of the most highly conserved and broadly expressed genes in multicellular eukaryotes. Recently, stress-induced NO-dependent S-nitrosation of *Arabidopsis* PRMT5 at Cys125 has been demonstrated, which increases the methyltransferase activity of this enzyme (Hu *et al.*, 2017). Enhanced S-nitrosation of PRMT5 in plants with loss-of-function mutations in S-nitrosogluthathione (GSNO) reductase (GSNOR) (Feechan *et al.*, 2005; Lee *et al.*, 2008b; Chen *et al.*, 2009), suggests that this enzyme is indirectly regulated by GSNOR activity, which controls global levels of GSNO, a natural NO donor. Importantly, through its effect on PRMT5 activity, NO modulates pre-mRNA splicing during plant stress. This might represent a novel post-transcriptional mechanism by which NO diversifies the stress-induced proteome through regulation of functional transcripts and formation of new splice variants mediated by S-nitrosation of PRMT5 (Frungillo & Spoel, 2017). Whether S-nitrosation of other PRMT Cys residues, Cys260 and Cys425 (Hu *et al.*, 2017), is biologically relevant, requires further investigation, in addition to how S-nitrosation might potentially affect other PRMT5 functions in plants, i.e. control of circadian rhythms (Hong *et al.*, 2010). Interestingly, rat PRMT1 is also under redox control through reversible oxidations of Cys residues to sulfenic acid by H₂O₂, resulting in concentration-dependent inhibition of methyltransferase activity (Begara-Morales *et al.*, 2015). Thus, in the wider context of redox signalling, it is intriguing to speculate that plant PRMTs might also be modulated by ROS.

Conclusions

It is now becoming apparent that a major route for NO bioactivity is through the manipulation of key PTMs, for example, SUMOylation, phosphorylation, persulfidation and acetylation (Fig. 2). By targeting key Cys residues, which function as regulatory redox switches, for oxidative modification, principally through S-nitrosation, NO is able to modulate the functions of these ubiquitous and fundamental PTMs, tailoring cellular responses to diverse challenges. The identification and subsequent characterisation of these strategically evolved redox switches will present exciting future opportunities to shape protein function towards

279 advantageous outcomes. For example, redox switches could be designed and implemented by
280 emerging gene editing strategies to potentially control a plethora of key biological processes
281 underpinning a variety of important agricultural traits. The ability of NO to regulate the
282 regulator may be at the heart of these new technologies.

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Table and figure legends

Table 1. Representative examples of enzyme involved in ROS metabolism whose activities are regulated by both NO and H₂S.

Figure 1. NO regulates SUMOylation through S-nitrosation of SUMO conjugating enzyme. (a) In the absence of nitric oxide (NO), SUMO (small ubiquitin-like modifier) conjugating enzyme (SCE1) SUMOylates key substrates with SUMO1/2 contributing to the repression of salicylic acid (SA)-dependent genes and by extension, the suppression of immunity in the absence of pathogens.

(b) Pathogen recognition triggers a nitrosative burst leading to NO accumulation, which results in the S-nitrosation of SCE1 at Cysteine (Cys)139. This redox based post-translational modification inhibits SCE1 activity blocking SUMO 1/2 SUMOylation. Consequently, this enables the expression of SA-dependent genes and the subsequent activation of plant immunity.

Figure 2. NO regulates a series of diverse post-translational modifications / signalling

systems. Integrative schematic representation of cross-talk between NO and various post-translational modification / signalling systems. A major route for NO bioactivity is through protein S-nitrosation (SNO) to form S-nitrosated proteins. NO-modified regulators modulate downstream processes through diverse chemical modification systems. Chemical modifications include ubiquitinylation (Ub), SUMOylation (S), phosphorylation (Pi), methylation (Me), acetylation (Ac), S-sulfenylation (SOH) and persulfidation (SSH). Plant functions regulated by these processes are indicated at the periphery of the diagram. ERFVII, group VII ethylene response factor; ERFVII-CR, arginylated ERFVII; HAD, histone deacetylase; PKs, protein kinases; PRMT5, protein arginine methyltransferase 5; RBOH, Respiratory burst oxidase homolog (NADPH oxidase); SCE1, SUMO E2 enzyme; SUMO, small ubiquitin-like modifier.